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Multiple Nuclear Loci Reveal the Distinctiveness of the Threatened, Neotropical *Pinus chiapensis*

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ABSTRACT. *Pinus chiapensis* is a threatened species of pine from southern Mexico and Guatemala. It was first described as a disjunct variety of *P. strobus* from the eastern United States and Canada. Subsequent morphological work indicates that *P. chiapensis* is a distinct species, but this interpretation is controversial. To explore the distinctiveness of this taxon, we sequenced three low-copy, unlinked nuclear loci in multiple accessions of *P. chiapensis* and its three most probable progenitors (*P. ayacahuite*, *P. monticola*, and *P. strobus*). *Pinus chiapensis* had the lowest combined nucleotide diversity of the four species (0.0031), and had only a single allele rangewide at one locus. *Pinus chiapensis* does not share alleles with any of the possible progenitors and all of its alleles are monophyletic at two of the three loci. At the third locus, allelic nonmonophyly is statistically indistinguishable from monophyly. While our results show that *P. chiapensis* is at least as distinct as the remaining three widely accepted species, determination of the most recent common ancestor is complicated by lack of allelic monophyly within potential progenitors and interlocus variability. Based on our sample of individuals and loci, *P. ayacahuite* appears to be the least likely progenitor, but there is no clear resolution of whether *P. chiapensis* is more closely related to *P. monticola* or *P. strobus*.

KEYWORDS: lineage sorting, nuclear phylogeny, Pinaceae, *Pinus strobus*, phylogenetic conflict.

Pinus chiapensis (Mart.) Andresen (= *Pinus strobus* var. *chiapensis* Mart., Pinaceae) ranges from Veracruz, Mexico south into northwestern Guatemala (Fig. 1). This species occurs in small remnant populations in the mountains of the Sierra Madre Occidental, Sierra Madre del Sur, Sierra Madre de Oaxaca, Sierra de los Chuchumatanes, and the highlands of Chiapas at elevations ranging from 260 – 2300 m (Dvorak et al. 1996). These habitats receive 1500 – 3000 mm of precipitation (Perry 1991; Farjon and Styles 1997). Associates include other species of pines as well as several species of broadleaved trees, some of which are considered disjunct from temperate counterparts in the eastern United States (Sharp 1953; Dressler 1954; Martin and Harrell 1957). These disjunctions are thought to date to the Pleistocene (Martin and Harrell 1957), and species showing similar disjunctions include *Liquidambar styraciflua* L. and *Carpinus caroliniana* Walt.

The conservation status of *P. chiapensis* is of increasing concern due to habitat loss and population fragmentation associated with human population expansion into the highlands, exploitation of this tree as a timber resource, and agricultural practices that clear land for crops (del Castillo and Acosta 2002). In addition, this species has a restricted range that is naturally fragmented due to past climatic change and discontinuous distribution of suitable habitat. The declining status of this taxon prompted the IUCN to list *P. chiapensis* as vulnerable (Farjon and Page 1999) and the FAO (Food and Agriculture Organization of the United Nations 1981) to list it as rare (small populations that are at risk) and endangered (extinction likely if causal factors continue unabated). While there have been no data reported on the specific loss of acreage for this taxon, its importance as a genetic resource has not been overlooked. For example, CAMCORE (Central America and Mexico Coniferous Resources Cooperative, North Carolina State University; Donahue et al. 1991) established *ex-situ* germplasm banks and provenance tests outside of the native range of this species beginning in 1984.

Pinus chiapensis is one of ca. 23 members of sect. *Quinquefoliae* (Gernandt et al. 2005), eight of which are native to North America. Among these eight species in sect. *Quinquefoliae*, three grow in Mexico: *P. ayacahuite* (southern Mexico into Central America; Fig. 1), *P. chiapensis* (southern Mexico into Central America; Fig. 1), and *P. strobiformis* (northern Mexico extending into the southwestern United States). There is disagreement as to whether a fourth species, *P. flexilis*, occurs in Mexico (Perry 1991; Farjon and Styles 1997). Of the remaining five North American members, four (*P. albicaulis*, *P.*

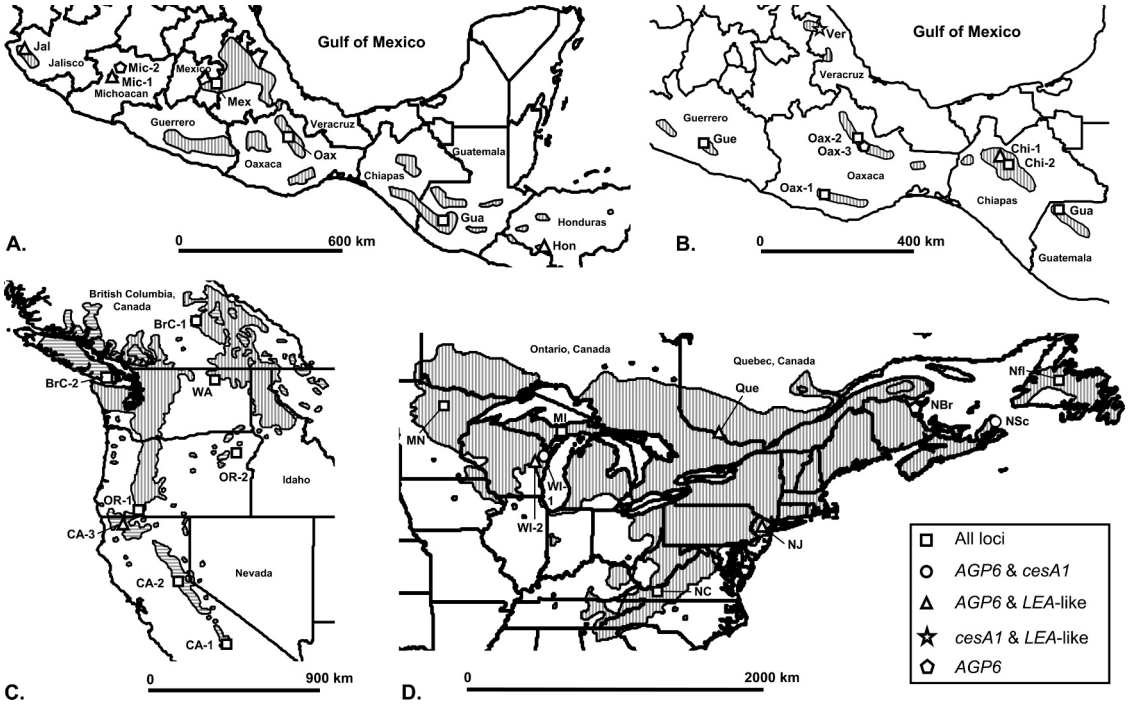

FIG 1. Distribution and sample locations for all accessions at all loci. *Pinus ayacahuite* (A), *P. chiapensis* (B), *P. monticola* (C), and *P. strobus* (D). Population codes are given in the collection table (Table 1). Ranges for *P. monticola* and *P. strobus* were taken from the U.S. Geological Survey (1999) and Critchfield and Little (1966). Distributions for *P. ayacahuite* and *P. chiapensis* were approximated from Perry (1991). For *P. ayacahuite,* distributions given by Perry (1991) for *P. ayacahuite* var. *veitchii* and *P. ayachahuite* were combined into a single map following Farjon and Styles (1997).

flexilis, P. lambertiana, and *P. monticola*) grow in the western United States and southwestern Canada. *Pinus strobus*, which grows in the eastern United States and southeastern Canada, is a notable outlier to this primarily western North American group (Fig. 1). *Pinus strobus* and *P. monticola* are considered to have the most similar morphology to *P. chiapensis* (Andresen 1966; Farjon and Styles 1997). *Pinus ayacahuite* is morphologically distinct, but is sympatric and could potentially hybridize with *Pinus chiapensis* (Perry 1991). All remaining North American pines in sect. *Quinquefoliae* are allopatric relative to *P. chiapensis* and are morphologically distinct.

Martínez (1940) first described *Pinus chiapensis* as a variety of *P. strobus*. Since the closest distance between occurrences in the United States and Mexico is ca. 2100 km (across the Gulf of Mexico) to 2400 km (shortest continental route), *P. strobus* var. *chiapensis* would represent a disjunct extension of the more extensive northern taxon. *Pinus chiapensis* shares a general morphological resemblance to *P. strobus,* having similar form, bark characteristics (furrowed bark breaking into long rectangular plates versus the distinctly square bark plates of *P. monticola*), cone size, and general cone morphology. Aside from morphological resemblance between *P. chiapensis* and *P. strobus*, the previously described floristic affinities between Mexico and the eastern U.S. appear to have influenced Martínez's decision to include *P. chiapensis* within *P. strobus* (Sharp 1953; Dressler 1954; Martin and Harrell 1957). In contrast, several authors (including Andresen 1966; Rzedowski and Vela 1966; Wright et al. 1996) make mention of the morphological similarities between *P. chiapensis* and *P. monticola.*

Gaussen (1960) was the first to propose elevating *P. chiapensis* to the level of species, but in failing to follow the conventional rules of nomenclature it was Andresen (1964) who is credited with this change. Andresen's evidence came from a multivariate morphometric analysis among *P. strobus*, *P. monticola*, and *P. chiapensis*, where it was determined that the latter was sufficiently distinct from the other species on the basis of multiple characters, including the number of reflexed scales contiguous to the peduncle, the number of serrations along the needle, needle width, and length to width ratio of the needles (Andresen 1966). Andresen also stressed the ecological differences between the tropical *P. chiapensis* and the temper-

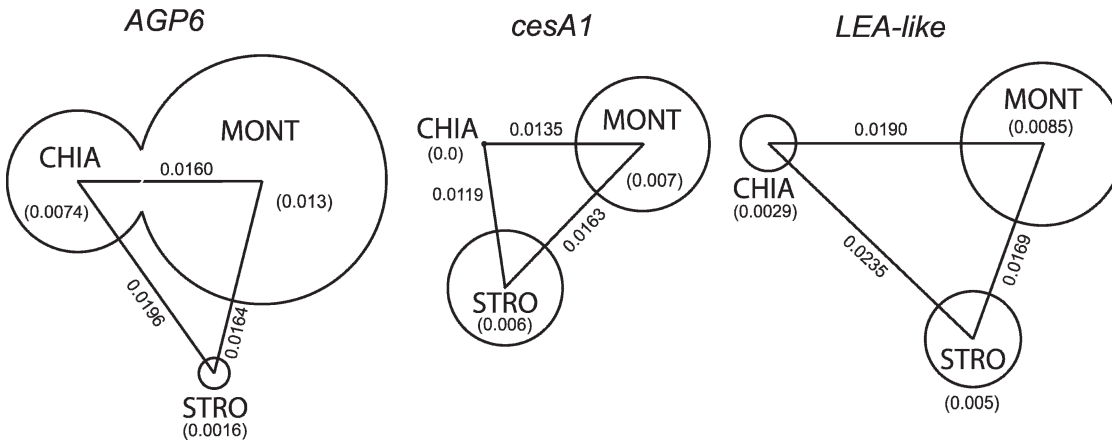

FIG 2. Graphic representation of within-taxa and between-taxa nucleotide diversity as measured by p-distances. Size of the circles represents within-taxa means (values in parentheses), lines connecting circles are between-taxa means (values given). CHIA = P. chiapensis, MONT = P. monticola, and STRO = P. strobus. All three loci are at the same relative scale.

ate *P. strobus* and *P. monticola*. Interestingly, Andresen (1966) reached the conclusion that the eastern *P. strobus* has a much closer morphological affinity to the western *P. monticola* than either has to *P. chiapensis*.

Not all taxonomists have followed Andresen in recognizing *P. chiapensis* as a distinct species. Among the taxonomic treatments that have considered these taxa, Perry (1991), Landry (1989), Price et al. (1998), and Gernandt et al. (2005) recognize *P. chiapensis*, but Critchfield and Little (1966), Little and Critchfield (1969), Kral (1993), and Farjon and Styles (1997) retain it at the varietal rank. In response to Andresen (1966), Farjon and Styles (1997) argue that the absence of discontinuities in traits among taxa in the morphometric analysis, and the lack of unique traits to *P. chiapensis*, represent a weak case for the recognition of this taxon as a distinct species.

Previously published molecular data regarding both the rank and affinity of *P. chiapensis* are conflicting and incomplete. Results from nrITS (Liston et al. 1999) do not support *P. chiapensis* and *P. strobus* as sister taxa, but statistical support for species-level relationships are essentially absent from nrITS based studies in pines. In addition, other potential progenitors (including *P. monticola*) were not sampled in that study. However, Liston et al. (1999) concluded that the substantial sequence divergence between *P. chiapensis* and *P. strobus* suggests that their isolation may predate the Pleistocene. Results from the cpDNA analysis of Gernandt et al. (2005) showed a weak sister relationship between *P. chiapensis* and *P. strobus* (52% bootstrap support [BS]; note that bootstrap values for *P. chiapensis* + *P. strobus* vs. *P. ayacahuite* + *P. flexilis* are transposed in Fig. 2 of Gernandt et al. 2005; D. Gernandt, pers. comm.). This resolution could be consistent with either the rank of variety (e.g., *P. strobus* var. *chiapensis*) or species (*P. chiapensis*). Significantly, cpDNA (Gernandt et al. 2005) shows *P. chiapensis* to resolve outside a well supported (99% BS) clade that contains *P. monticola.* Greater intraspecific sampling at the *matK* locus, which includes range wide samples of all North American members of subsection *Strobus*, supports the findings of Gernandt et al. (2005) and reveals a weakly supported (63% BS) sister relationship between *P. chiapensis* and *P. strobus*, but no sharing of haplotypes (Liston et al. 2007).

Recently, phylogenetic information gleaned from Late Embryogenesis Abundant (*LEA*)-like nuclear gene sequence was used to evaluate relationships among all soft pines (Syring et al., 2007). In that study, lack of allelic monophyly was shown for three of the eight North American representatives of sect. *Quinquefoliae,* and the authors concluded that incomplete lineage sorting was the primary factor responsible for the lack of monophyly. Of relevance to the present study, *P. chiapensis* and *P. strobus* both showed allelic monophyly at the *LEA*-like locus, while *P. monticola* showed significant nonmonophyly that is indicative of the retention of substantial ancestral diversity. At this locus, both alleles sequenced from *P. chiapensis* were strongly supported as monophyletic (100% BS), had minimal sequence divergence (p-distance = 0.0046), and were divergent from both *P. strobus* and *P. monticola*. Whether allelic monophyly is a general condition across the nuclear genome of *P. chiapensis* remains to be demonstrated. While *allelic* monophyly at a single locus is not necessarily an indication of *species* monophyly (Rosenberg 2003),

allelic monophyly across multiple loci could provide evidence of the genetic distinctiveness of a taxon (Baum and Donoghue 1995; Cronn et al. 2003; Small et al. 2004; Peters et al. 2005), and perhaps a clearer picture of its ancestral relationships with other pine species.

In this study we present data from three nuclear genes obtained from multiple accessions of four species from North American sect. *Quinquefoliae*: *P. ayacahuite*, *P. chiapensis*, *P. monticola*, and *P. strobus*. The goals of this effort were to address three specific questions. First, is the pattern of allelic monophyly indicated by *LEA*-like mirrored across other unlinked regions of the nuclear genome? Second, based on combined evidence, can molecular data reveal the most recent common ancestor (MRCA) of *P. chiapensis*? Lastly, how much genetic diversity is contained within *P. chiapensis*, and how does this compare to the other pine species that have been identified as possible progenitors of *P. chiapensis*?

Materials and Methods

Plant Materials. Seven species of *Pinus* subgenus *Strobus* were sampled (Table 1). This includes four North American species of subsect. *Quinquefoliae* and three species from subsect. *Gerardianae* which were used as the outgroup (*P. bungeana*, *P. gerardiana*, and *P. squamata*). *Pinus bungeana* was not sequenced for *AGP6*. Subsect. *Gerardianae* were used as the outgroup because data from Syring et al. (2007) indicate that the relationships among members of subsect. *Quinquefoliae* are unresolved. The number of sequences obtained for each ingroup species varies by locus, but ranges from three to ten (Table 1), with populations sampled from across their respective geographic ranges (Fig. 1). Sequences published in earlier studies (Syring et al. 2005, 2007) are indicated in Table 1.

Locus Amplification, Sequencing, Alignment, and Data Analysis. Three low-copy nuclear loci were sequenced in this study. The first, *AGP6,* has high sequence identity to an arabinogalactan-like protein that localizes to linkage group 5 in *P. taeda* L. (Krutovsky et al. 2004; GenBank AF101785) and is associated with secondary cell wall formation in differentiating xylem (Zhang et al. 2003). Primer sequences are modified from Syring et al. (2005; *AGP6* F2, 5′-GGTCAACAATGGCGTTCAAT; *AGP6* R2, 5′-TCTAACGTGAAGCGACAGGA) to include a larger portion of the gene.

The second locus sequenced in this study, *cesA1*, is part of the large cellulose synthase gene family that codes for the membrane-bound cellulose synthase (Richmond and Somerville 2000). Gene structure was inferred from comparisons between *Arabidopsis thaliana* (L.) Heynh. (GenBank AF458083) and *Pinus taeda* (GenBank AY789650), and primers were developed based on *P. taeda* sequence. The forward primer (CXF10: 5′-ATCCAAGGGCCAGTGTATGT) is located in the putative exon 10, and is 204 bp upstream of intron 10; the reverse primer (CX11R1: 5′-CAAACGACTTCTCAAAGCTTCTCT) lies 36 bp downstream of the 5′ end of exon 11.

The third locus shows high identity with a Late Embryogenesis Abundant (LEA)-like gene identified in *Pseudotsuga menziesii* (Mirbel) Franco (Iglesias and Babiano 1999; GenBank AJ012483). *LEA*-like is derived from a loblolly pine cDNA clone that maps to linkage group 3 in *P. taeda* (Krutovsky et al. 2004; C. S. Kinlaw, Institute of Forest Genetics, unpublished data; GenBank AA739606). Additional details of *AGP6* and *LEA*-like (maps of their structure, primer sequences used in amplification) are given in Syring et al. (2005).

DNA extraction, PCR amplification, and DNA sequencing followed the methods of Syring et al. (2005, 2007). In nearly all cases, haploid megagametophyte tissue was used as the source of DNA. In rare cases when needle tissue was used for DNA, direct sequencing identified mostly homozygotes, and only one heterozygote (New Jersey collection of *P. strobus* at *LEA*-like). These heterozygous PCR products were cloned into pGem-T Easy (Promega, Madison, Wisconsin). Alignments are available at TreeBASE (study number S1580). Analysis of sequences and the scoring of gaps followed Syring et al. (2007). Recombination was assessed using both the maximum χ^2 (Smith 1992; Posada and Crandall 2001) and sum of squares methods (DSS; McGuire et al. 1997; Milne et al. 2004) using the protocols outlined in Syring et al. (2007). To determine whether sample sizes are sufficiently large enough that random branching can be ruled out as the cause of allelic monophyly, we applied a statistical test that is based on the expectations of the coalescent process (Rosenberg 2007). The computed statistic indicates the probability that allelic monophyly could occur by chance within a specified lineage given the level of both intraspecific and interspecific sampling. The threshold level of significance for these tests was evaluated at $\alpha = 0.05$.

Phylogenetic analyses were performed by locus using maximum parsimony (MP; PAUP* version 4.0b10; Swofford 2002). Branch support was evaluated using the nonparametric bootstrap (Felsenstein 1985), with 1000 replicates and TBR branch swapping. Constraints on topologies were applied in PAUP* and the Wilcoxon signed-rank test of Templeton (WSR; Templeton 1983) was employed to test for significant differences among topologies. For this test, up to 1000 most-parsimonious trees recovered were used as constraint topologies. The range of *P* values across all topologies is reported in every case. When testing the constraint of species or lineage-specific monophyly, the lack of significance in the WSR tests indicates that nonmonophyly could be the result of insufficient phylogenetic signal.

Results

Sequence Characteristics of Low-Copy Nuclear Loci in Pinus. AGP6. From the *AGP6* locus we obtained 19 unique alleles from 34 sequences for the ingroup species (Table 2). The number of unique alleles ranged from three to six per species. From the Washington population of *P. monticola* and from the Jalisco population of *P. ayacahuite* we observed two alleles from one maternal parent (Table 1). Aligned *AGP6* sequences were 774 bp in length and corresponded to nucleotide positions 55-691 of *P. taeda* (GenBank AF101785). Individual sequences of the four ingroup species averaged 731 bp in length (range = 729 – 735 bp), while outgroup sequences were 774 bp in length. The aligned sequence included 227 complete codons and one partial codon at the 5′ end. The exon had 46 variable positions, of which eight were localized in first codon positions, eight in second positions, and 30 in third positions; replacements occur at 16 of 227 amino acid sites. Twenty-two of the variable

positions were parsimony informative (PI). The alignment included a 92-bp intron spanning nucleotide positions 632 – 723. The intron segment included eight variable sites and five PI sites. Nucleotide frequencies were relatively GC-rich (17.7% A, 19.5% T, 23.2% G, 39.6% C).

The intron had a single inferred indel at nucleotide position 642 that was shared between alleles of *P. ayacahuite* (Jal) and *P. monticola* (OR-1, WA, BC-1). Although *AGP6* was predominantly exonic, four indels occured within exons. One indel was fixed across the ingroup members relative to subsect. *Gerardianae*. Indels in the exon ranged from three to 39 nucleotides in length, and all maintained the reading frame. In total, five gaps were scored and appended to the *AGP6* alignment. One allele was shared across geographically diverse populations of two species: *P. ayacahuite* (Guatemala, Honduras, and Oaxaca, Mexico) and *P. strobus* (Michigan, North Carolina, New Jersey, Nova Scotia, and Quebec). All other alleles were restricted to a single species.

Estimates of within-taxa nucleotide diversity (π) in the *AGP6* data set ranged from 0.0016 (*P. strobus*) to 0.013 (*P. monticola*; Table 2). The relationship of within- and between-taxon nucleotide diversity is shown in Figure 2 for *P. chiapensis*, *P. monticola*, and *P. strobus*. *AGP6* was the only locus in this study where *P. chiapensis* was not strictly monophyletic; this is depicted in Figure 2, where *P. chiapensis* and *P. monticola* show overlapping allele pools as a result of large within-taxon and small between-taxon allelic divergence. Average genetic distances were smallest for *P. chiapensis* – *P. monticola* (0.0160), and greatest for *P. chiapensis* – *P. strobus* (0.0196). However, average genetic distances for *P. monticola* – *P. strobus* (0.0164) was nearly as small as for the comparison of *P. chiapensis* – *P. monticola*. Average genetic distances for *P. ayacahuite* – *P. chiapensis* (not shown in Fig. 2) was greater than all other comparisons at 0.0221.

CESA1. Using *cesA1,* we obtained 22 sequences for the ingroup taxa, representing 9 unique alleles, ranging from one to four per species (Table 2). Our aligned *cesA1* sequences were 1036 bp in length, and the average length of individual alleles for the four ingroup species was 1000 bp (range = 881 – 1032 bp), while outgroup species ranged from 1026 – 1029 bp. The alignment included 166 bp of exon, including 155 bp from exon 10 (54 complete codons) and 11 bp from exon 11 (3 complete codons). Exons showed three variable positions, two of which were parsimony informative, and all of which were localized to silent (third codon) positions. The intron included 36 variable sites and 33 PI sites. Nucleotide frequencies were AT-rich (31.8% A, 34.8% T, 16.6% G, 16.8% C).

Simple indels were moderately frequent across the length of the intron, and ranged from 1 – 151 nucleotides in length. No indels were present in the exon. In total, 20 gaps were scored and appended to the alignment. Three of the indels were shared across the species boundaries of the ingroup members. One indel was shared among all members of *P. chiapensis* and *P. strobus*, except the southernmost sample of *P. strobus* from North Carolina. No interspecific allele sharing was detected at this locus.

Estimates of within-taxon π range from 0.000 (*P. ayacahuite* and *P. chiapensis*) to 0.007 (*P. monticola*; Table 2). Between-taxon genetic distances were smallest for *P. chiapensis* – *P. strobus* (0.0119), intermediate for *P. chiapensis* – *P. monticola* (0.0135) and *P. monticola* – *P. strobus* (0.0163; Figure 2), and largest for *P. ayacahuite* – *P. chiapensis* (0.0153; not shown in Fig. 2).

LEA-LIKE From the *LEA*-like locus we obtained 18 unique alleles from 30 ingroup species (Table 2). The number of unique alleles ranged from three to seven per species. From the southern Oregon (OR-1) and northern Californian (CA-3) samples of *P. monticola*, we observed both alleles from the maternal parents (Table 1).

Our aligned sequence for the *LEA*-like locus was 990 bp in length. Average ingroup lengths were 894 bp (range = 858 – 941 bp), and outgroup sequences ranged from 847 – 980 bp in length. The alignment included 147 bp of exon from the 3′ end of the amplicons (positions 844 – 990), including 48 complete and two partial codons. The exon had four variable positions, of which one was at a second codon position and resulted in an amino acid replacement, while the remaining 3 were in silent (third) positions; none of these variable positions were parsimony informative. The intron segment included 51 variable sites and 30 PI sites. Nucleotide frequencies were slightly AT-rich (26.2% A, 33.1% T, 20.4% G, 20.3% C).

Complex and simple indels were moderately frequent across the length of the intron, and ranged from 1 – 65 nucleotides in length. No indels were present in the exon. In total, 18 gaps were scored and appended to the alignment. Two of the indels were shared across species boundaries of the ingroup members. A single indel was shared by all members of *P. chiapensis*, and another indel was shared between *P. bungeana* and all members of *P. chiapensis*. No interspecific allele sharing was detected.

Estimates of within-taxon π ranged from 0.0015 (*P. ayacahuite*) to 0.0085 (*P. monticola*; Table 2).

TABLE 1. Sampled *Pinus* subsects. *Gerardianae* and *Strobus* representatives. [a] Taxonomy follows Gernandt et al. (2005). [b] Published previously in Syring et al. (2007). [c] Accessions in parentheses represent multiple alleles sequenced from the same collection. [d] RILOG = Silva Tarouca Research Institute for Landscape and Ornamental Gardening, 252 43 Průhonice, Czech Republic. [e] United States Department of Agriculture Forest Service. na = no sample for this locus.

Taxon[a]	Code	GenBank (*AGP6, cesA1, LEA-like*)[c]	Collection	Collector or Source (Voucher)
Section *Quinquefoliae*				
Subsection *Gerardianae* (Outgroup)				
P. bungeana Zuccarini ex Endlicher		na; DQ898470; DQ642500[b]	Shanxi Province, China	USDAFS[e] Institute of Forest Genetics (OSC)
P. gerardiana Wallich ex D. Don	*P. gerardiana*-1	DQ898468; na; na	Gilgit, Pakistan	Businský 41105 (RILOG[d])
	P. gerardiana-2	na; DQ898471; DQ642498[b]	Gilgit, Pakistan	Businský 41123 (RILOG[d])
P. squamata X.W. Li	*P. squamata*-1	DQ898469; DQ898472; na	Yunnan, China	Businský 46118 (RILOG[d])
	P. squamata-2	na; na; DQ642501[b]	Yunnan, China	Businský 46120 (RILOG[d])
Subsection *Strobus* (Ingroup)				
P. ayacahuite Ehrenberg ex Schlechtendal	Mex	DQ898434; DQ898492; DQ642450[b]	Mexico, Mexico	USDAFS Institute of Forest Genetics (OSC)
var. veitchii (Roezl) Shaw	Jal	(a-DQ898435, b-DQ898436); na; DQ8985504	Jalisco, Mexico	USDAFS Institute of Forest Genetics (OSC)
	Mic-1	DQ898437; na; DQ642451[b]	Michoacan, Mexico	USDAFS Institute of Forest Genetics (OSC)
P. ayacahuite Ehrenberg ex Schlechtendal	Hon	DQ898438; na; DQ642452[b]	La Paz, Honduras	USDAFS Institute of Forest Genetics (OSC)
	Gua	DQ898439; DQ898493; DQ898505	Totonicapa, Guatemala	USDAFS Institute of Forest Genetics (OSC)
	Oax	DQ898440; DQ898494; DQ898506	Oaxaca, Mexico	USDAFS Institute of Forest Genetics (OSC)
	Mic-2	DQ898441; na; na	Michoacan, Mexico	Alejandra Moreno-Letelier (no voucher)
P. chiapensis (Martínez) Andresen	Chi-1	DQ898442; na; DQ642453[b]	Chiapas, Mexico	Dvorak, CAMCORE (no voucher)
	Gua	DQ898443; DQ898473; DQ642454[b]	Guatemala	USDAFS Institute of Forest Genetics (OSC)
	Ver	na; DQ898474; DQ642455[b]	Veracruz, Mexico	Hernandez (OSC)
	Oax-3	DQ898444; na; na	Oaxaca, Mexico	Gernandt DSG00999 (MEXU)
	Gue	DQ898445; DQ898475; DQ898500	Guerrero, Mexico	Syring 1012 (OSC)
	Oax-1	DQ898446; DQ898476; DQ898501	Oaxaca, Mexico	Syring 1018 (OSC)
	Oax-2	DQ898447; DQ898477; DQ898502	Oaxaca, Mexico	Syring 1020 (OSC)
	Chi-2	DQ898448; DQ898478; DQ898503	Chiapas, Mexico	Syring 1022 (OSC)
P. monticola Douglas ex D. Don	OR-1	DQ898449; DQ898479; (a-DQ898507, b-DQ642462[b])	Oregon, USA	USDAFS Dorena Genetic Resource Center (OSC)
	WA	(a-DQ898450, b-DQ898451); DQ898480; DQ898508	Washington, USA	USDAFS Dorena Genetic Resource Center (OSC)
	CA-3	DQ898452; na; (a-DQ898509, b-DQ898510)[d]	California, USA	USDAFS R5 Camino Seed Orchard (OSC)
	OR-2	DQ898453; DQ898481; DQ898511	Oregon, USA	USDAFS Dorena Genetic Resource Center (OSC)
	BrC-1	DQ898454; DQ898482; DQ898512	British Columbia, Canada	Natural Resources Canada (OSC)
	BrC-2	DQ898455; DQ898483; DQ898513	British Columbia, Canada	Natural Resources Canada (OSC)
	CA-1	DQ898456; DQ898484; DQ642464[b]	California, USA	USDAFS Central Zone Genetic Resource Program (OSC)
	CA-2	DQ898457; DQ898485; DQ898514	California, USA	USDAFS Central Zone Genetic Resource Program (OSC)

TABLE 1. Continued.

Taxon[a]	Code	GenBank (*AGP6*, *cesA1*, *LEA-like*)[c]	Collection	Collector or Source (Voucher)
P. strobus Linnaeus	WI-1	DQ898458; DQ898486; na	Wisconsin, USA	USDAFS Dorena Genetic Resource Center (OSC)
	WI-2	DQ898459; na; DQ642468[b]	Wisconsin, USA	USDAFS Dorena Genetic Resource Center (OSC)
	MI	DQ898460; DQ898487; DQ898495	Michigan, USA	USDAFS Oconto River Seed Orchard (OSC)
	MN	DQ898461; DQ898488; DQ642469[b]	Minnesota, USA	USDAFS Oconto River Seed Orchard (OSC)
	Que	DQ898462; na; DQ898496	Quebec, Canada	Canadian Forest Service (OSC)
	NSc	DQ898463; DQ898489; na	Nova Scotia, Canada	Natural Resources Canada (OSC)
	NBr	DQ898464; na; na	New Brunswick, Canada	Natural Resources Canada (OSC)
	Nfl	DQ898465; DQ898490; DQ898497	Newfoundland, Canada	Natural Resources Canada (OSC)
	NC	DQ898466; DQ898491; DQ898498	North Carolina, USA	USDAFS National Tree Seed Laboratory (OSC)
	NJ	DQ898467; na; DQ898499	New Jersey, USA	Gernandt DSG00500 (OSC)

TABLE 2. Summary of the number of sequences (N) and the number of unique alleles (A) acquired per species across all three loci. Within group estimates of nucleotide diversity (π) and standard deviation (SD) were calculated from p-distances using 500 bootstrap replicates. Combined π was weighted by average sequence length across all three loci, and a rank was assigned from 1 (highest average π) to 4 (lowest average π).

	AGP6				*CESA*				*LEA*-like				Combined	
Species	N	A	π	SD	N	A	π	SD	N	A	π	SD	π	Rank
P. ayacahuite	8	5	0.011	0.0022	3	1	0	0	6	3	0.0015	0.0008	0.0036	3
P. chiapensis	7	3	0.0074	0.0021	6	1	0	0	7	3	0.0029	0.0012	0.0031	4
P. monticola	9	6	0.013	0.0022	7	4	0.007	0.0017	10	7	0.0085	0.0018	0.0092	1
P. strobus	10	5	0.0016	0.0007	6	3	0.006	0.0016	7	5	0.005	0.0014	0.0044	2

Between-taxon genetic distances were smaller for *P. chiapensis* – *P. monticola* (0.0190) than for *P. chiapensis* – *P. strobus* (0.0235; Figure 2). However, the mean distance between *P. monticola* – *P. strobus* (0.0169) was 11% smaller than the *P. chiapensis* – *P. monticola* value. Mean genetic distances between *P. ayacahuite* – *P. chiapensis* was on the same order as the *P. chiapensis* – *P. strobus* comparison (0.0227).

Tests of Recombination and the Coalescent Process. No evidence of recombination among the included sequences was detected using either the maximum χ^2 method or the DSS method. As a result, no sequences were excluded from the analyses on the basis of being recombinant products. With our level of sampling of species and allele lineages, monophyly arising as a consequence of random branching is unlikely for all loci ($P = 0.018$ for *cesA1*, $P \leq 0.01$ for *AGP6* and *LEA*-like).

Phylogenetic Analyses. AGP6. From the branch and bound search of the *AGP6* data set, one most parsimonious tree was recovered (Fig. 3). The tree was 107 steps in length, had a consistency index (CI) of 0.785, and a retention index (RI) of 0.872. *AGP6* was the only locus in which the alleles for *P. chiapensis* were not exclusively monophyletic, but instead were paraphyletic with one allele from *P. monticola* (CA-1; southern extent of the range) nested within the weakly supported (68% bootstrap support; BS) ''*P. chiapensis*'' clade. *Pinus chiapensis* alleles appeared to be divided into two clades with moderate support (76% BS); one including alleles from Oaxaca and Chiapas, and the other including *P. chiapensis* alleles from populations in Guatemala, Guerrero, and Oaxaca, and the allele from *P. monticola* (CA-1). In a constraint analysis forcing the monophyly of *P. chiapensis*, WSR results were insignificant (Table 3), indicating that the CA-1 allele of *P. monticola* was not statistically supported inside the ''*P. chiapensis*'' clade. In contrast, the nonmonophyly of all *P. monticola* alleles was statistically supported in the *AGP6* phylogeny (Table 3). Interestingly, three of the six *P. monticola* alleles (representing six of the nine populations) were strongly supported as monophyletic (91% BS) in the most parsimonious tree. These three alleles represented the northern diversity of *P. monticola*, ranged from Oregon through British Columbia, and were weakly supported (55% BS) as sister to the ''*P. chiapensis*'' clade. The three *P. monticola* alleles not in this clade were all from Californian populations. Aside from the *P. monticola* allele found in the ''*P. chiapensis*'' clade, the other two Californian alleles were in a weakly supported (54% BS) or unsupported position as sister to either alleles of *P. ayacahuite* or *P. ayacahuite* + *P. strobus*, respectively.

The only detected case of allele sharing between species occurred at the *AGP6* locus between *P. strobus* and *P. ayacahuite*. In this case, the allele in common was shared among five populations of *P. strobus* and three populations of *P. ayacahuite* that have a combined geographic range spanning Nova Scotia, Canada to Honduras (2650 – 4450 km apart). All of the diversity of *P. strobus* was contained within a very strongly supported clade (99% BS) that included three of five *P. ayacahuite* alleles. Mean genetic distances between eight of the alleles from the *P. strobus* + *P. ayacahuite* clade was a minimal 0.26%, compared to 1.6% for all five of the *P. ayacahuite* alleles, 1.0% for all three *P. chiapensis* alleles, or 1.8% for all six of the *P. monticola* alleles. Constraining the alleles from *P. strobus* to be monophyletic resulted in a tree of equivalent length to the unconstrained tree, while constraining the alleles from *P. ayacahuite* to be monophyletic resulted in a highly significant WSR result (Table 3).

CESA1. A branch and bound search of the *cesA1* data set produced two most parsimonious trees that differed in the resolution among outgroup taxa (Fig. 3). Trees were 84 steps in length, had a consistency index (CI) of 0.905, and a retention index (RI) of 0.929. No allelic diversity was detected among the six *Pinus chiapensis* sequences taken from populations across its range. The recovered allele had five autapomorphic mutations

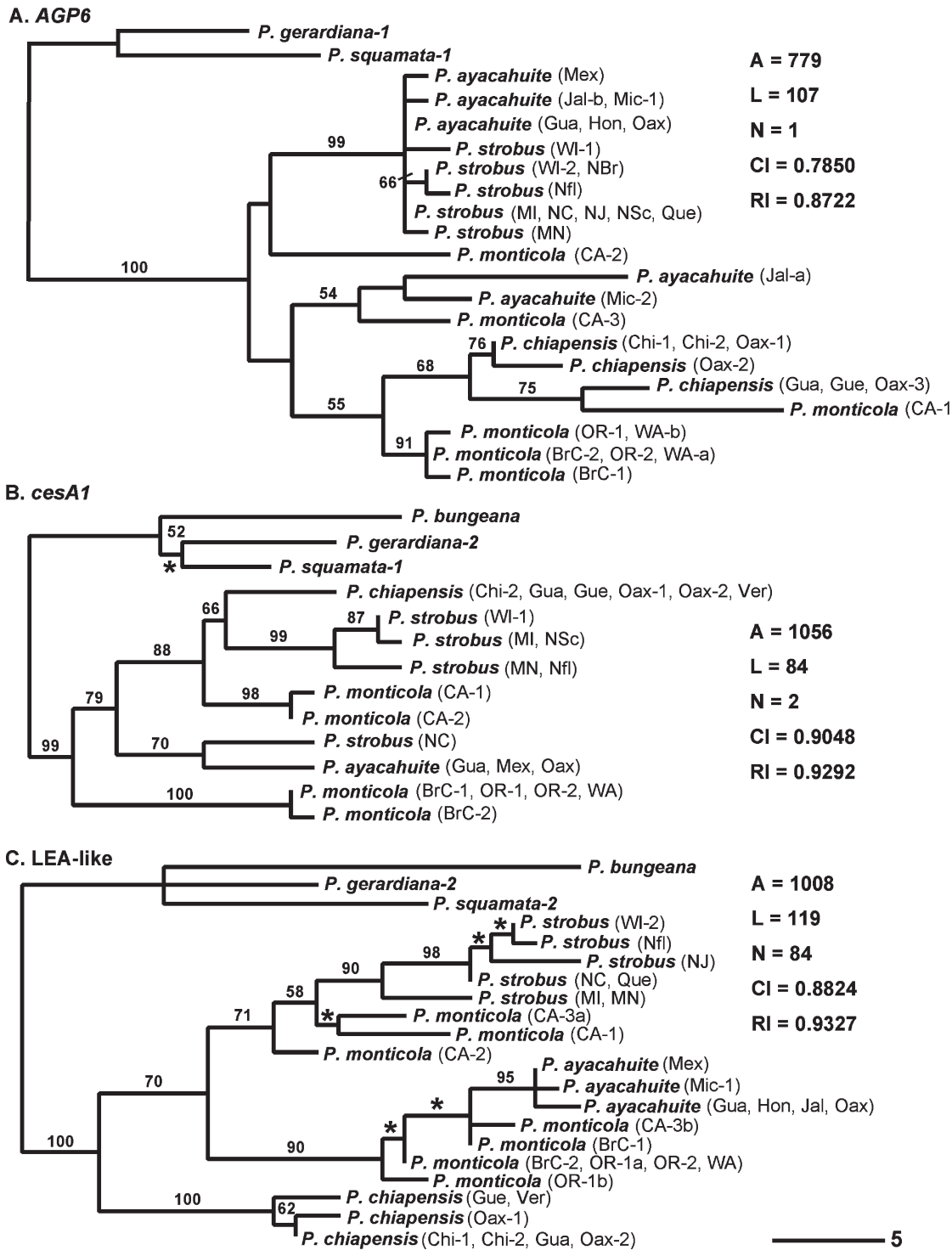

FIG 3. Most parsimonious trees derived from individual loci. Bootstrap values from 1000 replicates and TBR branch swapping are shown near nodes. A = aligned length of data set, L = length of trees, N = number of trees, CI = consistency index, and RI = retention index. Asterisks (*) indicate nodes that collapse in the strict consensus tree. Population codes refer to Table 1 and Figure 1.

TABLE 3. Wilcoxon signed rank (WSR) results from the topological constraint of monophyly enforced on either individual species or species pairs. All alleles from each species are used in each test. The level of significance was assessed at alpha = 0.05. Insignificant values indicate that the constrained topologies are not statistically different from the unconstrained trees. Boxed values are at least partially insignificant. Lu is the length of the unconstrained topologies shown in Figure 3. Lc is the length of the topology constrained for monophyly of *P. chiapensis* with the species of interest. N is the number of constrained trees recovered from a heuristic search. † In this case, 76.9% of the recovered topologies are significant, while 23.1% are insignificant.

Locus	Monophyly Constraint	Lu	Lc	N	Significance
AGP6	*P. ayacahuite*	107	117	3	0.0016
	P. chiapensis	107	110	2	0.3173, 0.3657
	P. monticola	107	117	5	0.0323 – 0.0388
	P. strobus	107	107	1	1.0000
	P. ayacahuite – P. chiapensis	107	122	9	0.0011 – 0.0046
	P. monticola – P. chiapensis	107	112	1	0.0253
	P. strobus – P. chiapensis	107	118	2	0.0165
	P. monticola – P. strobus	107	123	17	0.0007 – 0.0011
cesA1	*P. monticola*	84	89	1	0.0588
	P. strobus	84	86	2	0.1573, 0.3173
	P. ayacahuite – P. chiapensis	84	90	16	0.0143 – 0.0339
	P. monticola – P. chiapensis	84	91	2	0.0082, 0.0196
	P. strobus – P. chiapensis	84	86	6	0.1573 – 0.3173
	P. monticola – P. strobus	84	91	5	0.0196
LEA-like	*P. monticola*	119	127	4	0.0325 – 0.0455
	P. ayacahuite – P. chiapensis	119	128	84	0.0027 – 0.0067
	P. monticola – P. chiapensis	119	131	36	0.0005 – 0.0013
	P. strobus – P. chiapensis	119	125	52	0.0143 – 0.0578†
	P. monticola – P. strobus	119	123	12	0.2482 – 0.2850

relative to a shared node with three of the four alleles of *P. strobus*. The *P. chiapensis* lineage was weakly supported as sister to three of the four *P. strobus* alleles, which were very strongly supported (99%) as monophyletic. However, the sister relationship of *P. chiapensis* + *P. strobus* was based on a single synapomorphic position and is poorly supported (66% BS). Collapsing this poorly supported node yielded a strongly supported (88% BS) trichotomy that included *P. chiapensis,* three monophyletic alleles from *P. strobus* (99% BS), and the two monophyletic (98% BS) alleles from Californian *P. monticola*. The fourth *P. strobus* allele (NC), representing the southernmost sample from this study, was moderately supported (70%) as the sister to a single allele shared between the three populations of *P. ayacahuite*. The northern samples of *P. monticola* were very strongly supported as monophyletic (100% BS) and moderately supported as sister to the remaining diversity of the ingroup taxa (79% BS). The lack of species-level monophyly for the alleles of either *P. monticola* or *P. strobus* was not statistically supported in WSR constraint analyses (Table 3).

LEA-LIKE. From the branch and bound search of the *LEA*-like data set, 84 most parsimonious trees were recovered (Fig. 3). Trees were 119 steps in length, had a consistency index (CI) of 0.882, and a retention index (RI) of 0.932. The topology of the strict consensus of the 84 most parsimonious trees matched the *LEA*-like tree shown in Figure 3, except that nodes with less than 58% BS collapsed. The three alleles representing seven populations of *P. chiapensis* were strongly supported (100% BS) as monophyletic, and shared eight synapormorphies that separated them from a moderately supported (70% BS) clade of *P. ayacahuite, P. monticola,* and *P. strobus.* Within the *P. chiapensis* clade, the alleles from Veracruz and Guerrero were identical. Of the two remaining alleles, accessions from Chiapas-1, Chiapas-2, Guatemala, and Oaxaca-2 shared the same allele, and were weakly supported (62% BS) in a clade with the Oaxaca-1 allele. The alleles for *P. ayacahuite* were very strongly supported (95% BS) as monophyletic and were nested in a strongly supported (90% BS) clade that contained the northern-most accessions of *P. monticola*. The alleles for *P. strobus* were strongly resolved as monophyletic (90% BS), and were nested within a clade defined by Californian *P. monticola* (71% BS). Constraining the alleles of *P. monticola* to be monophyletic resulted in a significant WSR result (Table 3).

Topological Tests of the MRCA of Pinus chiapensis. Across all data sets topological constraints were enforced for the monophyly of all *P. chiapensis* alleles with the alleles for each of the three other ingroup species (Table 3). Forcing the monophyly of *P. chiapensis* with each alternative species yielded only two insignificant results (α =

0.05), implying that a sister relationship cannot be statistically rejected in those two cases. The insignificant results both involved *P. strobus*, one at the *cesA1* locus and the other at *LEA*-like. However, the latter result was insignificant in only 12 of the 52 total trees (23.1%), while the remaining 40 constrained trees were significant. Further, constraining *LEA*-like topologies for the monophyly of *P. monticola* – *P. strobus* resulted in 12 most parsimonious trees that were all not significant in a WSR analysis. If significance in Table 3 is assessed at the $\alpha = 0.01$ level, then a sister relationship cannot be rejected for *P. chiapensis* and either *P. monticola* or *P. strobus* at *AGP6*, all three alternative species and *P. chiapensis* at *cesA1* (*P. monticola* for 1 of 2 trees), and *P. chiapensis* and *P. strobus* for all recovered topologies at the *LEA*-like locus.

Discussion

Recognition of Pinus chiapensis at the Taxonomic Rank of Species. Based on the combined evidence from three nuclear loci, the distinctiveness of *P. chiapensis* warrants its recognition as a species. If *P. chiapensis* was a variety of any of the potential parent species sampled, the expectation is that alleles from *P. chiapensis* would either be shared across species boundaries or perhaps form a monophyletic clade that renders the parental species paraphyletic. We find that *P. chiapensis* is monophyletic at two loci (*cesA1*, *LEA*-like) and nearly so at a third (*AGP6;* Table 3). This trend of monophyly by *P. chiapensis* is mirrored by *P. strobus* (monophyletic at *LEA*-like, nearly so at *cesA1*) and *P. ayacahuite* (monophyletic at *LEA*-like and *cesA1*), but contrasts *P. monticola* which is polyphyletic at all loci. Coalescent expectations indicate that it is highly unlikely for stochastic processes alone to result in the allelic monophyly observed in *P. chiapensis* at *LEA*-like and *cesA1* (Rosenberg 2007). This trend towards species monophyly is contrasted by the inconsistent resolution of *P. chiapensis* across loci; sister to *P. monticola* at *AGP6*; sister to *P. strobus* at *cesA1*; and sister to all three potential parent species at *LEA*-like. The absence of a consistent resolution makes it impossible to determine the sister taxon to *P. chiapensis*, but from the perspective of allelic monophyly, this taxon is at least as distinct as the other sampled species, all of which are recognized at the specific rank.

In addition to the molecular evidence, Andresen's (1966) multivariate analysis provides morphological support for the distinctiveness of *P. chiapensis*. Andresen identified three traits that provided the greatest discrimination of *P. chiapensis* from either *P. monticola* or *P. strobus*; the number of reflexed basal-scales contiguous to the peduncle (*P. chiapensis* has no scales that are fully reflexed); the number of serrations per 5 mm interval at the needle center (most numerous in *P. chiapensis*); and the length to width ratio of the needles, with *P. chiapensis* having the smallest value and the ''finest'' or ''thinnest'' needles of the triad. For differentiating *P. chiapensis* from *P. strobus,* the number of involutions of edges of central scales are also significantly different, with *P. chiapensis* having 2.5 compared to 1.1 for *P. strobus*.

Finally, there are ecological and physiological differences that distinguish *P. chiapensis* from the near-relatives included in our study. *Pinus chiapensis* is a tropical pine distributed exclusively in humid, frost-free areas in Mexico and western Guatemala (del Castillo et al. 2004). By contrast, *P. strobus* and *P. monticola* are distributed in temperate forests where below freezing temperatures are common during winter. Similarly, *P. ayacahuite* is frost resistant and is distributed above the frost line in humid forests of Mexico and Central America. Perhaps as a physiological adaptation to its tropical distribution, the seeds of *P. chiapensis* do not require a cold stratification for germination (del Castillo et al. 2004).

Identifying a MRCA of Pinus chiapensis. Lack of species monophyly among the potential ancestors of *P. chiapensis*, most likely resulting from incomplete lineage sorting (Syring et al. 2007), and interlocus variability are both confounding factors in determining the MRCA of *P. chiapensis*. While gene tree parsimony (Slowinski and Page 1999) and the methods of Maddison and Knowles (2006) attempt to resolve conflict between discordant gene trees, these approaches do not provide statistical tests to determine support for the reconciled 'species' tree. Based on our sampling (3 loci, roughly 5 individuals per species, estimating tree depth at 1 N_e), simulations following Maddison and Knowles (2006) predict that their methods would result in a $< 60\%$ chance of obtaining the true tree. Rather than attempt to simplify the underlying complexity that characterizes closely-related species of pines, we chose to analyze our data gene by gene. In the future, coalescent based approaches may provide the greatest chance of resolving interlocus discrepancies driven by the process of incomplete lineage sorting.

Based on our data, the least likely common ancestor from the evaluated species appears to be *P. ayacahuite*. In phylogenetic analyses (Fig. 3), the alleles from *P. chiapensis* are never found in a sister relationship with alleles from *P. ayacahuite.* At both

the *cesA1* and *LEA*-like loci, *P. chiapensis* alleles are separated from *P. ayacahuite* alleles by multiple nodes with greater than 70% BS; at the *AGP6* locus the alleles from the two species are mutually exclusive and separated by five nodes (with the clades containing the *P. chiapensis* alleles having 75 – 76% BS). Mean genetic distances for *P. ayacahuite* – *P. chiapensis* are greater than remaining comparisons involving *P. chiapensis* at the *AGP6* and *cesA1* loci, while at *LEA*-like this comparison is very similar to that of *P. chiapensis* – *P. strobus* . Further, constraining topologies for the monophyly of *P. ayacahuite* – *P. chiapensis* results in significant WSR results at all three loci (Table 3). While these two species have overlapping ranges across Mexico, contact between these two related species is usually confined to the margins of their respective areas (del Castillo et al. 2004). It is possible that isolating mechanisms (phenology, and possibly pre-or post-zygotic incompatibilities) limit genetic exchange between these two species. The striking case of allele sharing at *AGP6* between *P. strobus* (from Newfoundland and North Carolina) and *P. ayacahuite* (from Oaxaca through Honduras) also underscores the low genetic affinity between *P. chiapensis* and *P. ayacahuite.* Allele sharing between *P. strobus* and *P. ayacahuite* reflects either ancestral retention of polymorphism (i.e. incomplete lineage sorting) or ancient introgression. This pattern is not evident in *P. ayacahuite* and *P. chiapensis,* highlighting the rarity of hybridization between these species and their distant coancestry.

Relationships among *P. chiapensis*, *P. monticola*, and *P. strobus* are more difficult to establish due to conflicting results across tests and loci. At *LEA*-like, tree topologies place *P. chiapensis* sister to all remaining species, and genetic distances between taxa (Figure 2) show *P. chiapensis* being more similar to *P. monticola* (π = 0.0190) than to *P. strobus* (π = 0.0235). However, constraining the topologies for the monophyly of *P. chiapensis* – *P. strobus* resulted in 23.1% of the topologies being insignificant, and thus not statistically different from the most parsimonious trees. In results that mirror the conclusions of Andresen (1966), *P. monticola* and *P. strobus* appear to be more closely related at *LEA*-like than either is to *P. chiapensis*. Average genetic distances between these two species (0.0169; Fig. 2) is smaller than for any comparison involving *P. chiapensis*, and constraining topologies for the monophyly of *P. monticola* – *P. strobus* is insignificant for all recovered trees in a WSR test (data not shown).

At *cesA1*, between-taxon genetic distances (Fig. 2) place *P. chiapensis* closer to *P. strobus* (π = 0.0119) than to *P. monticola* (π = 0.0135), and constraining topologies for the monophyly of *P. chiapensis* – *P. strobus* results in all six recovered trees being insignificant in a WSR analysis (Table 3). In contrast to the *LEA*-like locus, *cesA1* shows *P. monticola* and *P. strobus* to be more divergent from each other than either is to *P. chiapensis*. This result is supported in a WSR analysis where topologies constrained to the monophyly of *P. monticola* – *P. strobus* were significant for all recovered trees (Table 3). While this may seem like potential evidence for a *P. chiapensis* – *P. strobus* relationship, it was noted earlier that if nodes with less than 70% BS were collapsed in the most parsimonious trees that a trichotomy of *P. chiapensis, P. strobus* (minus the NC allele), and the Californian *P. monticola* alleles (CA-1, CA-2) would form a single well supported (88% BS) clade.

One finding of this study is that genetic diversity for *P. monticola* populations from California is consistently greater across all loci compared to populations north of the California/Oregon border. These results mirror the allozyme data of Steinhoff (1983). As a consequence, it may be more appropriate to consider southern *P. monticola* as the most probable ancestor to *P. chiapensis,* rather than *P. monticola* species-wide. In this light, it is interesting that the sequence from the southernmost extent of the range of *P. monticola* (CA-1) renders *P. chiapensis* paraphyletic at *AGP6*. At this locus, between-taxon comparisons of genetic diversity were greater for *P. chiapensis* – *P. strobus* than for either *P. chiapensis* – *P. monticola* or *P. monticola* – *P. strobus*. Looking at *AGP6* alone, one might reach the conclusion that *P. monticola* shares a MRCA of *P. chiapensis*.

However, when data from all loci are compared it seems apparent that insufficient information is available to make a determination with any degree of certainty as to the MRCA of *P. chiapensis*. Ruling out *P. ayacahuite* as the nearest relative of *P. chiapensis* is an important first step given their geographic proximity and potential for historical contact. Given the abundance of genomic resources available for pines, it is feasible to address the phylogenetic history of *P. chiapensis* across many loci and individuals, not only in the nuclear genome, (Krutovsky et al., 2006), but also at highly variable loci in the chloroplast (Shaw et al., 2005) and mitochondrial genomes. By exploring patterns of evolution across genes and genomes, it will be possible to address phylogeographic hypotheses, such as whether *P. chiapensis* came to occupy its current range in southern Mexico and Guatemala by long-distance dispersal, or via connections with

a northern or extinct relative during periods of favorable climate (Sharp 1953; Dressler 1954; Martin and Harrell 1957)..

Genetic Diversity of Pinus chiapensis. Loss of genetic diversity and heterozygosity are relevant conservation concerns for *P. chiapensis* due to the strong inbreeding depression exhibited by many pine species (Williams and Savolainen, 1996; Sorensen, 1999; Sorensen, 2001). In this regard, it is noteworthy that *P. chiapensis* has the lowest nucleotide diversity averaged across the three loci for the four species included in this study (Table 2). In addition, this species ranks among the lowest of all eight North American *Pinus* subsection *Strobus* species (Syring, unpublished data), and all Subg. *Strobus* species at the *LEA*-like locus (Syring et al., 2007). Similar findings have been made for nuclear loci using using isozymes (del Castillo, unpublished data), and cpDNA loci at the *matK* locus (Liston et al. 2007).

Estimates of genetic diversity are relevant to conservation concerns for *P. chiapensis* and other rare pines. In Syring et al. (2007), nucleotide diversity at *LEA*-like was estimated for 33 species from 2 – 3 alleles per species, and these estimates were compared to current species ranges (a proxy for census population sizes and global abundance). The conclusion was that genetic diversity and geographic ranges (or census sizes, approximately) were uncorrelated. For example, *P. bhutanica* Grierson, Long & Page has a relatively small geographic range for pines (ca. 8000 km^2 across SE Asia; roughly equivalent to *P. chiapensis*–5000 km^2), but it ranks in the top third with respect to genetic diversity (π = 0.0185). Similarly, *P. culminicola* Andresen & Beaman (from Mexico) and *P. dalatensis* de Ferré (from Vietnam) are known to occupy extremely small ranges (50 km^2), but they rank near the median of 33 species for genetic diversity (π = 0.0075 and 0.0077, respectively; Syring et al., 2007). While conservation efforts often focus on species with small or fragmented ranges, retention of genetic diversity may be of paramount importance in genetically depauperate species, particularly if they show strong inbreeding depression as has been described for pines (Sorenson 1999). In this light, *Pinus chiapensis* may be unusual among pines as it is apparently threatened by two key factors, a small and decreasing natural range, and unusually low genetic diversity.

At this point, a definitive cause for the low genetic diversity found in *P. chiapensis* would be highly speculative. However, given the theoretical relationship between genetic diversity (θ) and effective population size (N_e; e.g., $N_e \approx \theta/[4 \times \mu_G]$, where μ_G is the per-generation mutation rate; Tajima, 1983), it seems likely that *P. chiapensis* must have undergone a significant reduction in its N_e, possibly in the recent past. The events that created this bottleneck may be the actual origin of the *P. chiapensis* lineage (perhaps coupled with long-distance dispersal), or subsequent bottlenecks associated with naturally changing climatic conditions in the Holocene that have fragmented a larger population into higher-elevation islands. In contrast to other pine species that have more expansive ranges and diversity, human activities (over-exploiration of timber resources, additional habitat fragmentation and reduction) may exacerbate the severity of this historical bottleneck. The exceedingly low diversity present in this species underscores the need for immediate study into the effect of inbreeding on growth traits and reproductive fitness, as well as the importance of *in-situ* and *ex-situ* conservation of this ecologically and economically valuable resource.

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